Figure 1A

SEQ ID NO:1

/translation="MGSVLSTDSGKSAPASATARALERRRDPELPVTSFDCAVCLEVL
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DRSLLEYVNHSNTT"

Figure 1B

SEQ ID NO:2

AGCGGAGGTCATTTTTGCAGCTTATTGTGATGACAACAGTGGAGGATGGTCTTCCACTTCA
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Figure 1C

SEQ ID NO:3

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1801 ggcagaagaa tcgcttgagc ccgggaggcg gaggttgcag tgagctgaga tcgcgccagt
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```

Figure 1D

SEQ ID NO:4

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Figure 1E

SEQ ID NO:5

TRAC1 genomic region:

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2

Figure 1F

SEQ ID NO:6
Mouse TRAC1 cDNA sequence:

TGCGCGCGCTCCGCCTGCGCTCCACCGAGAGGCCCCGCGGCGCGGCTGGC
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TGAGCTAGAGCGCAAGNNTGGGCTCCCTGCTGAGCAGCGACAGCTCCAAG
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SEQ ID NO:7
Mouse TRAC1 protein (3rd frame)

SAXXGSLLSSDSSKSAPASATPRTLERSGDSELPITSFDCSVCLEVLHQP
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YQNCAECGTLVCLSDMRAHIRTCEKYIDKYGPLLELGDTTARCVCPFCQR
ELDEDCLLDHCIIHHRSERRPVFCPLCHSRPDESPSTFNGSLIRHLQVSH
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FIGURE 2A

FLJ20456 Hit Inhibited anti-TCR Induced CD69 Expression in Jurkat Cells
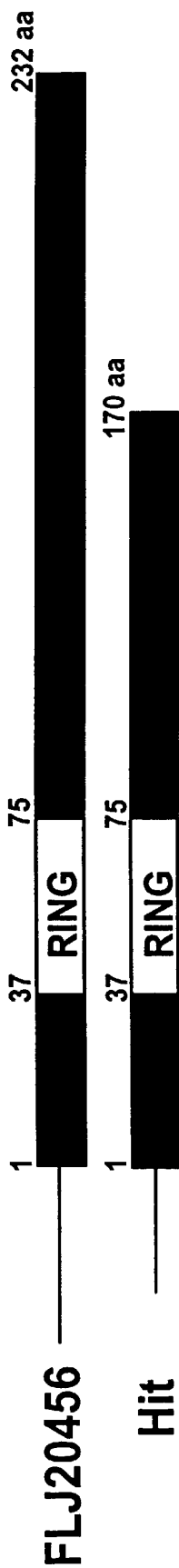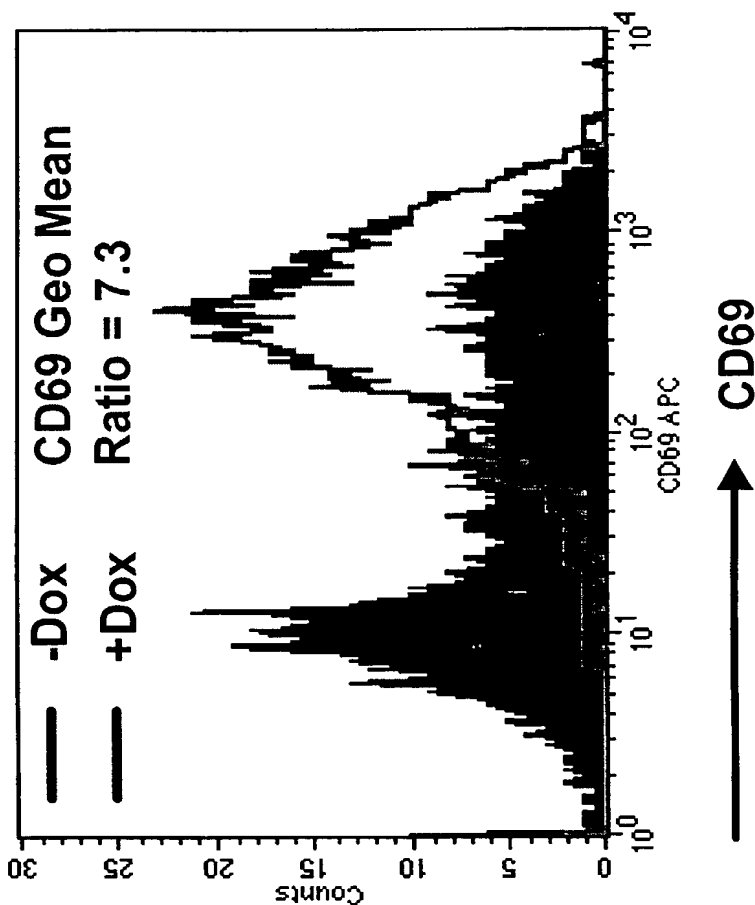
FIGURE 2B

FLJ20456 Hit Inhibited anti-TCR Induced CD69 Expression in Jurkat Cells Phenotype Transfer

Figure 3A

Full Length FLJ20456 Does Not Inhibit CD69 Upregulation in Jurkat Cells

FLJ20456

1   37   RING   75   232 aa

- Pfu PCR product amplified from a capped human brain cDNA library.
- One N to S polymorphism with FLJ20456 NM_017831.1 at amino acid 186, present in EST database.

JurkatN 32H

PC031801.010

R1

SSC

0   200 400 600 800 1000

FSC

0 200 400 600 800 1000

PC031801.010

PI

R2

Counts

0   40   80 120 160 200

FL3-Height

10^0 10^1 10^2 10^3 10^4
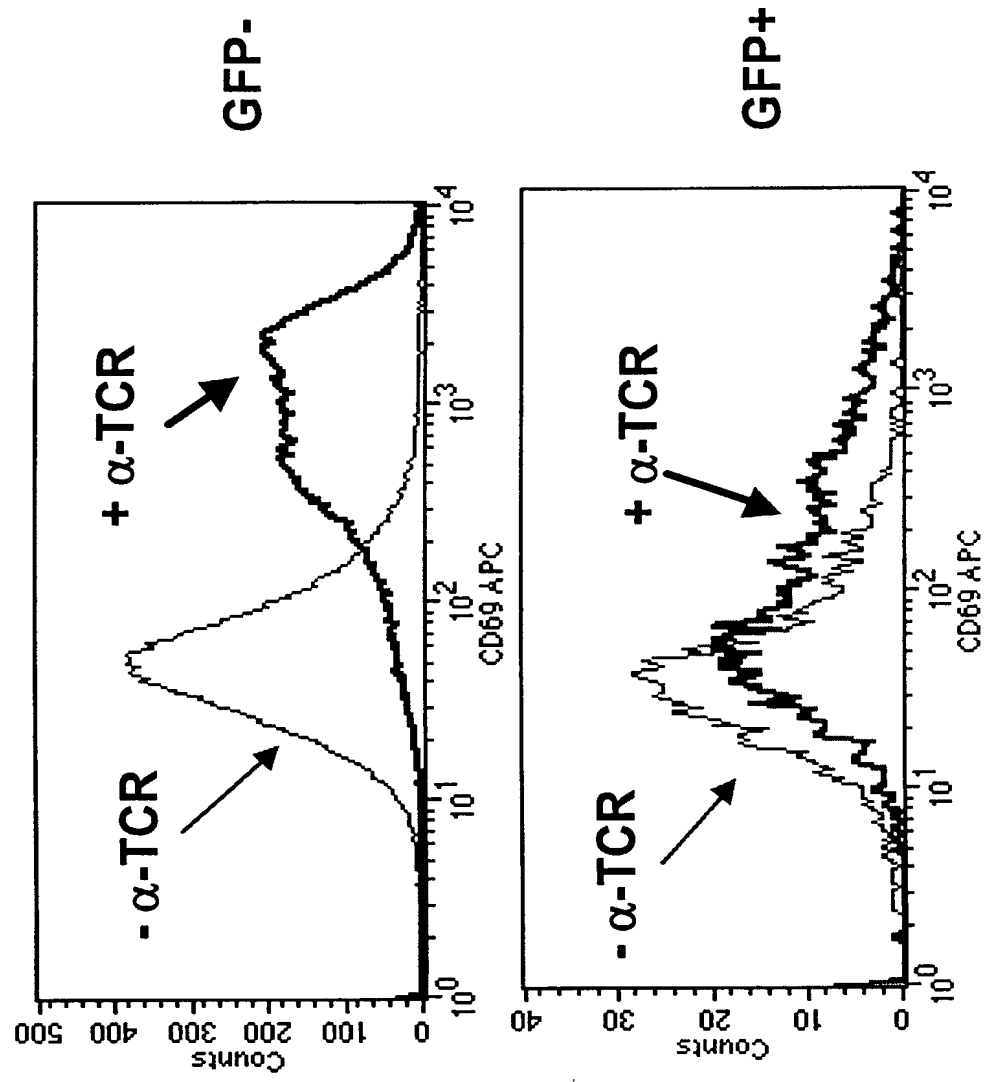
Figure 3B

Full Length FLJ20456 Does Not Inhibit CD69 Upregulation in Jurkat Cells

300ng/ml C305

GFP-   1070.5

GFP+   1219.9

Ratio = 0.88
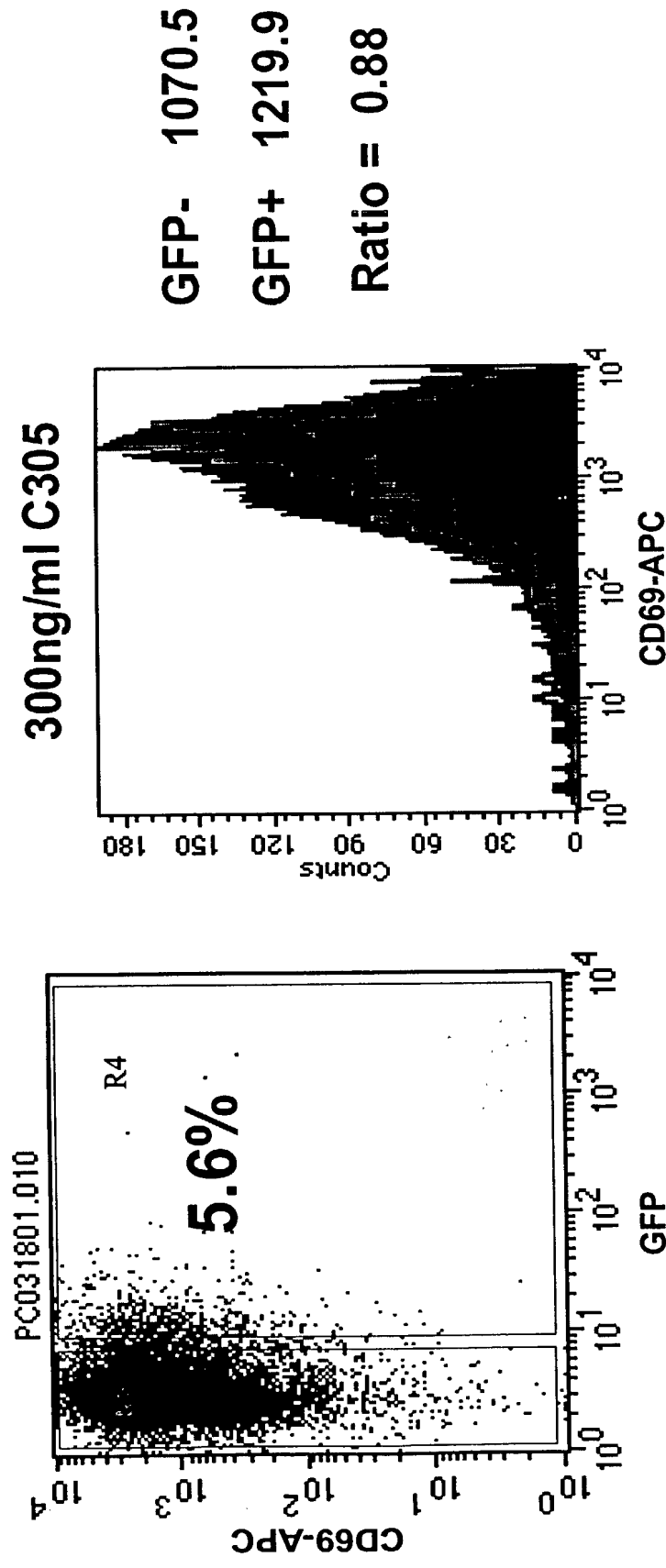
Figure 4A

FLJ20456 Hit Specifically Inhibited T Cell Activation but not B Cell Activation

FLJ20456

```
1        37  RING  75                              232aa
```

Hit

```
1        37  RING  75              170aa
```

Figure 4B

Figure 4C

Jurkat

Counts (300, 240, 180, 120, 60, 0)

CD69-APC (10^0, 10^1, 10^2, 10^3, 10^4)
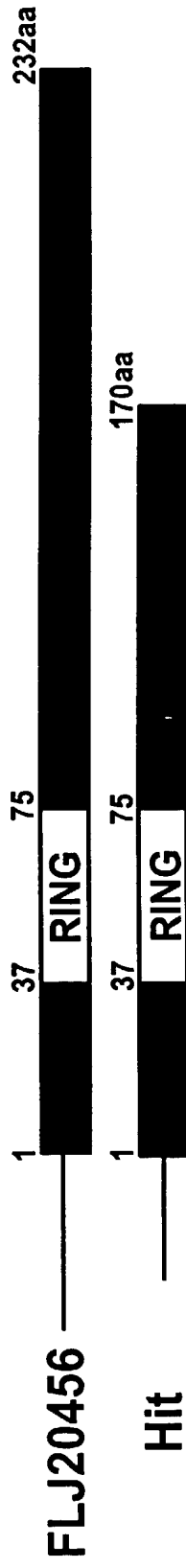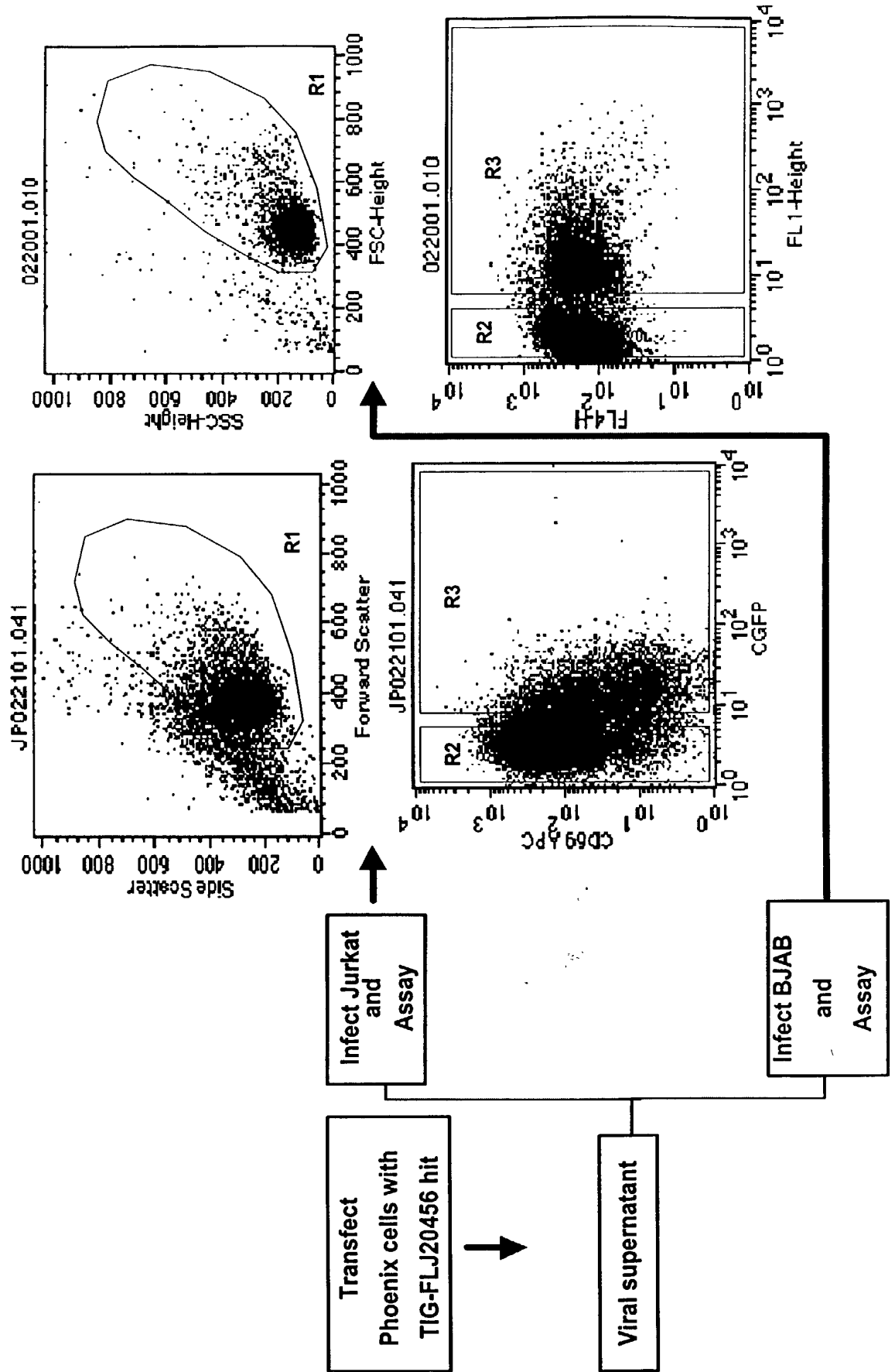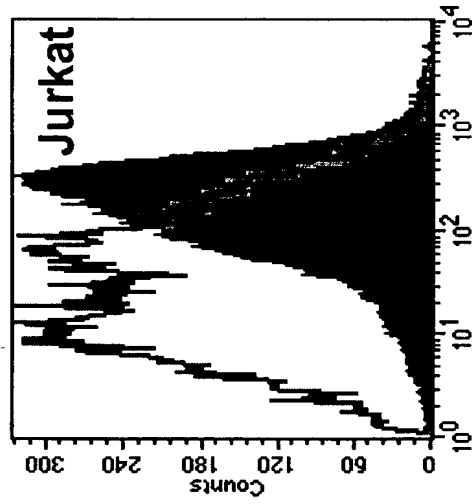
Stimulation:
300ng/ml C305

GFP-   141.2
GFP+   28.8
Ratio = 4.9

BJAB

Counts (100, 80, 60, 40, 20, 0)

CD69-APC (10^0, 10^1, 10^2, 10^3, 10^4)
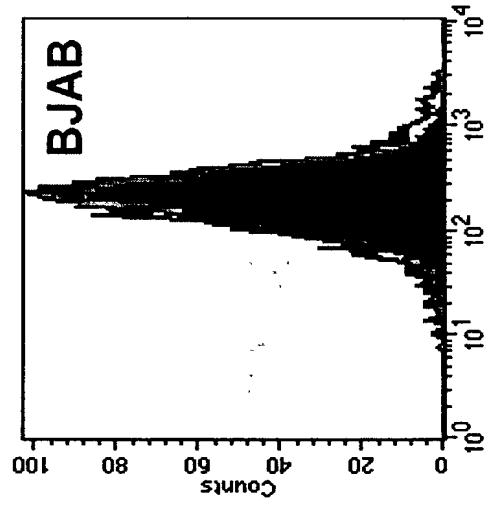
Stimulation:
300ng/ml α-IgM

GFP-   183.1
GFP+   183.3
Ratio = 1.0

Figure 5

FLJ20456 Is Strongly Expressed in Lymphoid and Hematopoietic Organs

Probe: FLJ20456 Hit

- FLJ20456 is expressed in multiple tissues
- The strongest expression is in PBL, Liver and Spleen
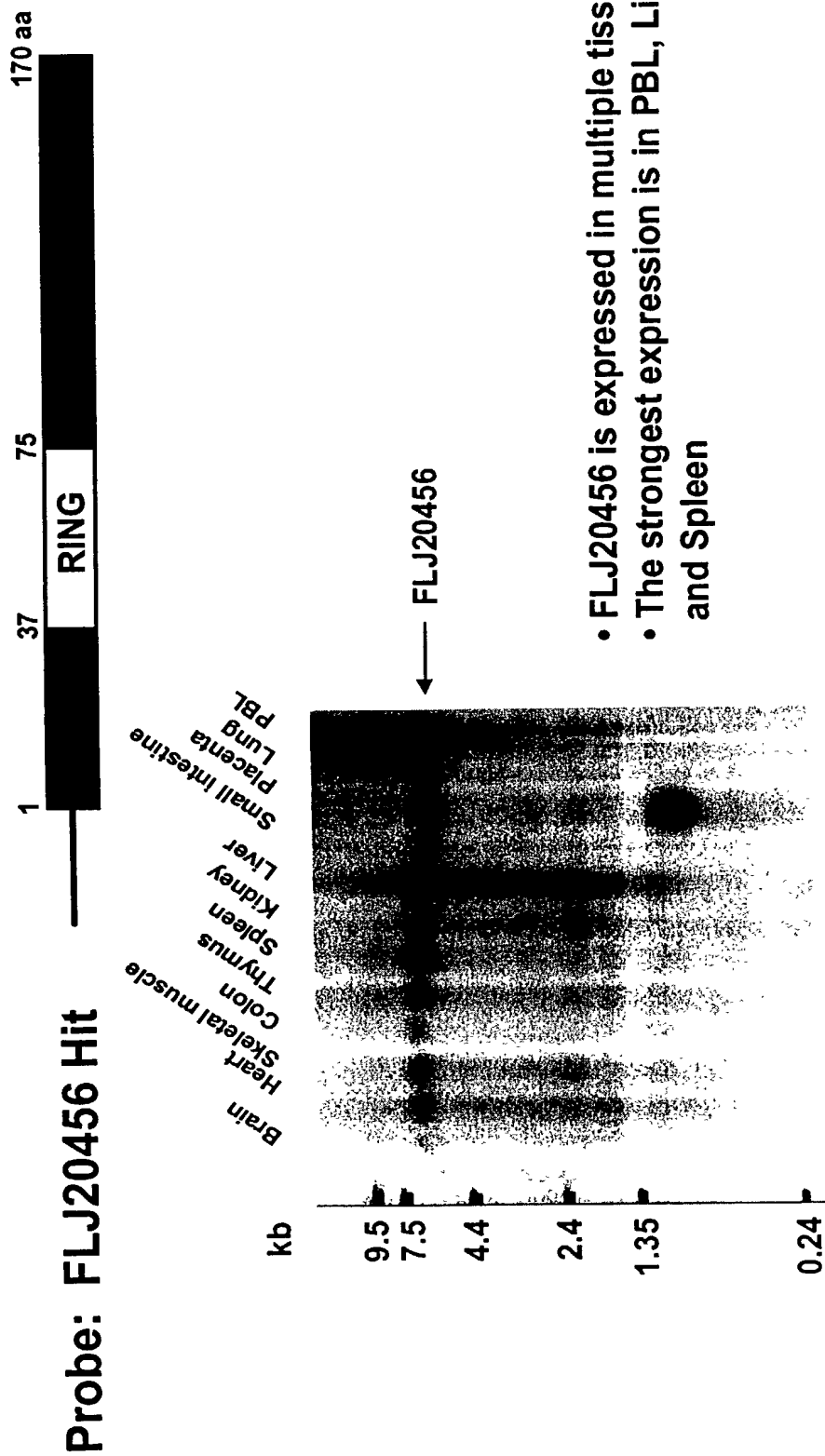
Figure 6

TRAC1 is Predominantly Expressed in Resting T lymphocytes

FLJ20456 Sequence is Most Similar to Two Sequences: Znf313 and STRIN

                                                    Ring Domain

Consensus #1 ...........................F.C.VC.EV...PV....C.HVFC...C.........

FLJ20456.pep MGSVLSTDSGKSAPASATARAIERRRDPELPVTSFDCAVCLEVIHQPVR-IRCGHVFCRSCIATSLKN 67
znf313.pep   ------MAAQQRDCGGAAQLAGPAAEADPLGRFTCPVCLEVYEKPVQV-PCGHVFCSACLQECLKP   59
STRIN.pep   ------MAEDLSAATSYTEDDEYCPVCQEVIKTPVRTACQHVFCRKCFLTAMRE   49

Consensus #1 ....C...CR..........A..........C.C.......R.H...C.KY...Y......

FLJ20456.pep NKWTCBYCRAYLPSE----GVPATDVAKRMKSEYKNCAECDTIVCLSEMRAHIRTCQKYIDKYGPLQE 131
znf313.pep   KKPVCGVCRSALAP-----GVRAVELERQIESTETSCHGCRKNFFLSKIRSHVATCSKYQN-YIMEGV 121
STRIN.pep   SGAHCDLCRGNVTRRERACPERALDLENIMRKFSGSCRCCAAKQIKFYRMRHYKSCKYQDEYGVSSI   117

Consensus #1 ............CP.C.........L.HC...H.....

FLJ20456.pep LEETAAR-------------------------CVCPFCQRELYE-DSLLDHCITHHRSERR   166
znf313.pep   KATIKDASLQPRNVPNRY--------TFPCPYCDKNFDQEGIVEHCKLFHSTDTK   169
STRIN.pep   VPNFQISQDSVGNSNRSETSTSDNTETYQENTSSSGHPTFKCPLCQESNETRQRLLDHCNSNHLFQIV 185

Consensus #1 .V.CP.C....P....P.....N...H.....Y.F.....EE..........S...........-

FLJ20456.pep PVFCPLCRLIPDENPSFSGNLIRHLQVSHTLFYDDFIIDFNIIEEALIRRVLDRSLLEYVNHSNTT. 233
znf313.pep   SVVCPICASMPWGDPNYRSANFREHIQRRHRFSYDTFVDYDVDEEDMMNQVLQRSIIDQ.   229
STRIN.pep   PMTCPICWSLPWGDPSQITRNEVSHINQRRQEDYGEEMNLQIDEETQYQTAVEESFQVNI.   246

	1	2	3	
1		26.6	22.3	1	FLJ20456.pep
2	130.4		27.9	2	znf313.pep
3	140.9	134.7		3	STRIN.pep
	1	2	3		

* All three sequences are human
* Murine sequences are not shown

FIG. 7.

Alignment of RING Domain Sequences from Various Human Proteins

```
Consensus #1    ......C..C.........C.H.C.........C.........

FLJ20456.Ring   VTSFDCAVCLEVLHQPVRTR-CGHVFCRSCIATSL--KNNKWTCPYCRAYLP-S   50
znf313.Ring     LGRFTCPVCLEVYEKPVQVP-CGHVFCSACLQECL--KPKKPVCGVCRSALA-P   50
STRIN.Ring      EDDFYCPVCQEVLKTPVRTTACQHVFCRKCFLTAM--RESGAHCPLCRGNV--T   50
TRAF6.Ring      ESKYECPICLMALREAVQTP-CGHRFCKACIIKSI--RDAGHKCPV-DNEILLE   50
c-Cbl.Ring      STFQLCKICAEN-DKDVKIEPCGHLMCTSCLTSW--QESEGQGCPFCRCEI-KG   50
BRCA1.Ring      --LECPICLELIKEPVSTK-CDHIFCKFCMLKLLNQKKGPSQCPLCKNDITKR   50
BAR.Ring        VSEFSCHCCYDILVNPT-TLNCGHSFCRHCLALWWA-SSKKTECPECREK--WE   50
RAG1.Ring       VKSISCQICEHILADPVETN-CKHVFCRVCILRCL--KVMGSYCPSCRYPCF-P   50
```

RING

FLJ20456

TRAF6 — MATH

Cbl — MATH — UBI A

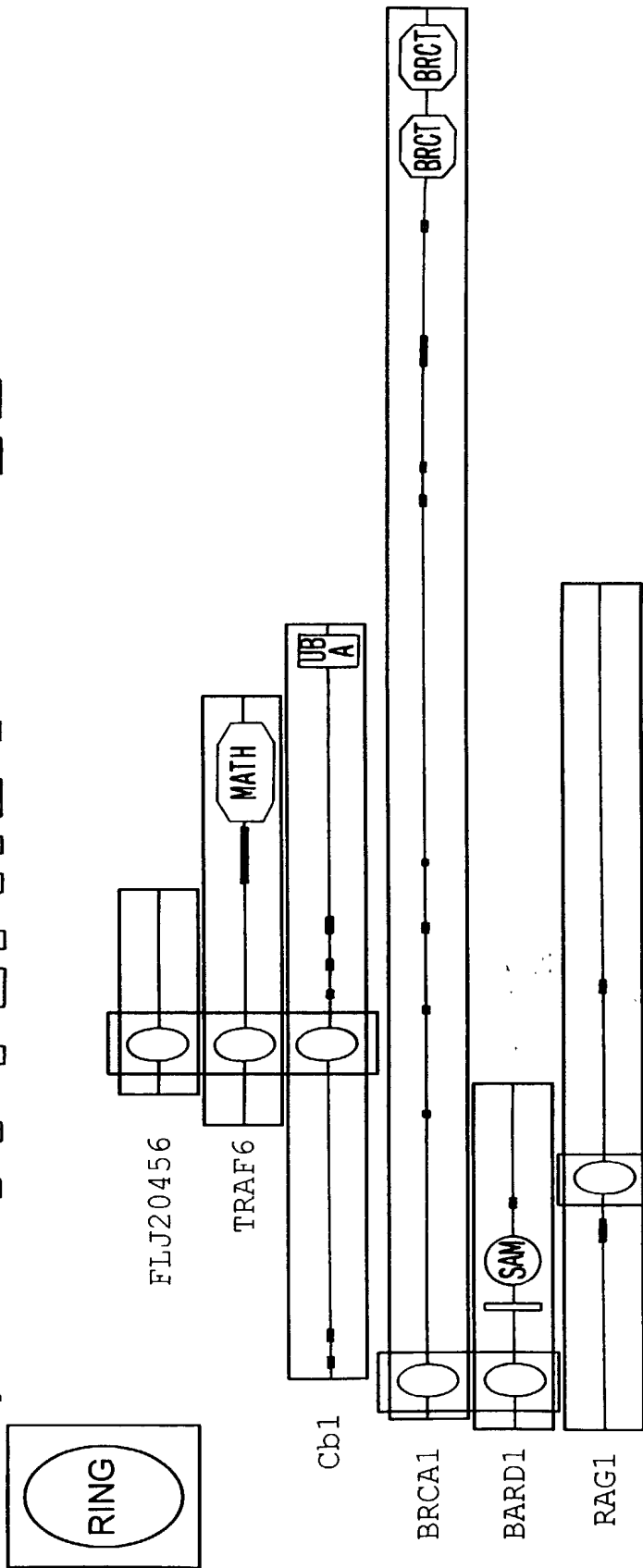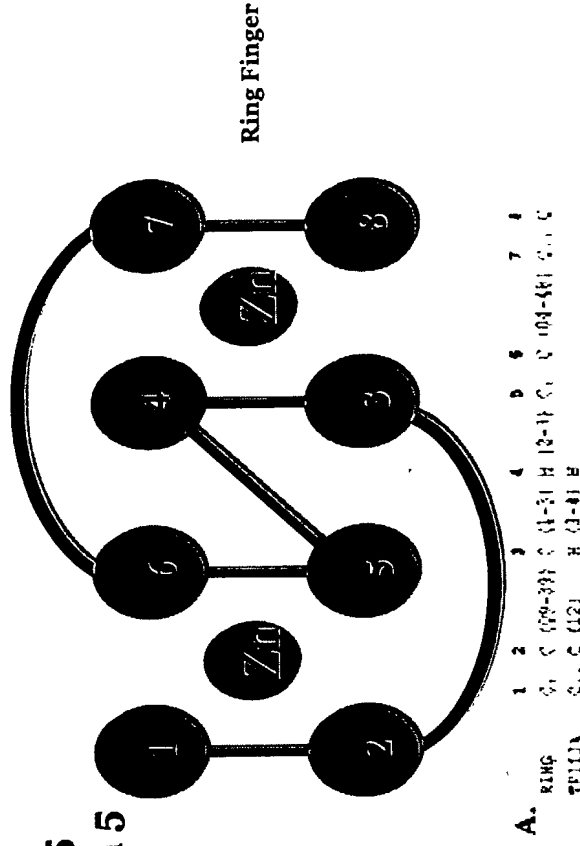BRCA1 — BRCT BRCT

BARD1 — SAM

RAG1

FIG. 8.

Figure 9

RING finger vs. Zinc finger proteins

Ring-HC: C_3HC_4 = Cys in position 5
Ring H2: C_3H2C_3 = His in position 5

- Ring finger domains have a conserved pattern of Cys and His residues that co-ordinate two zinc atoms to form a cross-brace structure
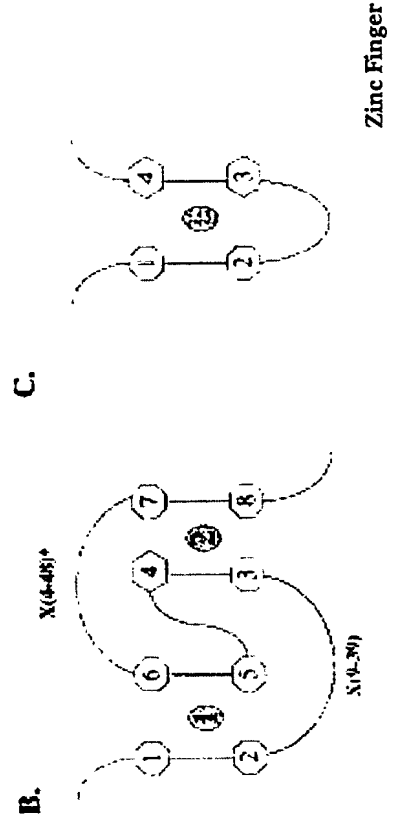
- Ring fingers are structurally distinct from zinc fingers

Ring Finger

Zinc Finger

A.

B.

C.

Figure 10A

Ubiquitin Pathway Components

- **E1:** ubiquitin-activating enzyme, with a major isoform that may work broadly

- **E2:** ubiquitin-conjugating enzyme, a class of ~14 enzymes, interacts with E3

- **E3:** ubiquitin ligases, a broad and growing group of activities that promote addition of ubiquitin to specific proteins

- **Proteasome**–a 26S complex containing a 19S lid and base that mediates ATP- and ubiquitin-chain-dependent binding of substrates and a 20S catalytic core with three known proteolytic activities.
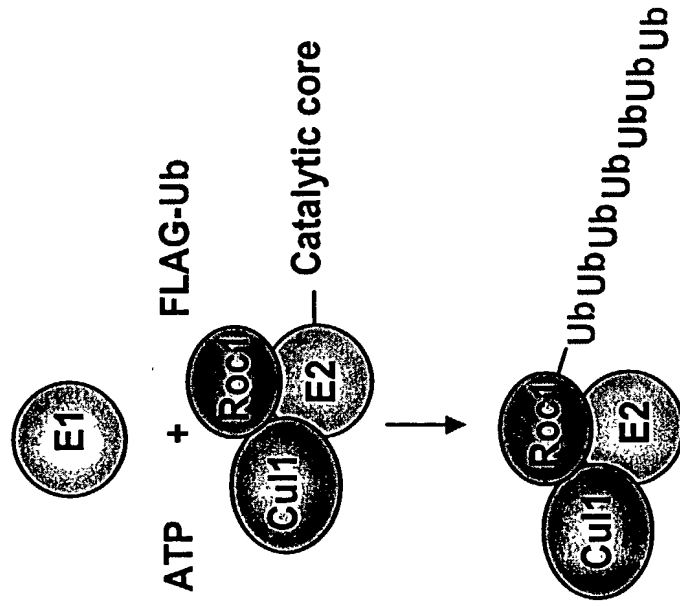
Figure 10B

Enzymology of Ubiquitination

Ub = ubiquitin	E2 = ubiquitin conjugating enzyme
E1 = ubiquitin activating enzyme	E3 = ubiquitin ligase
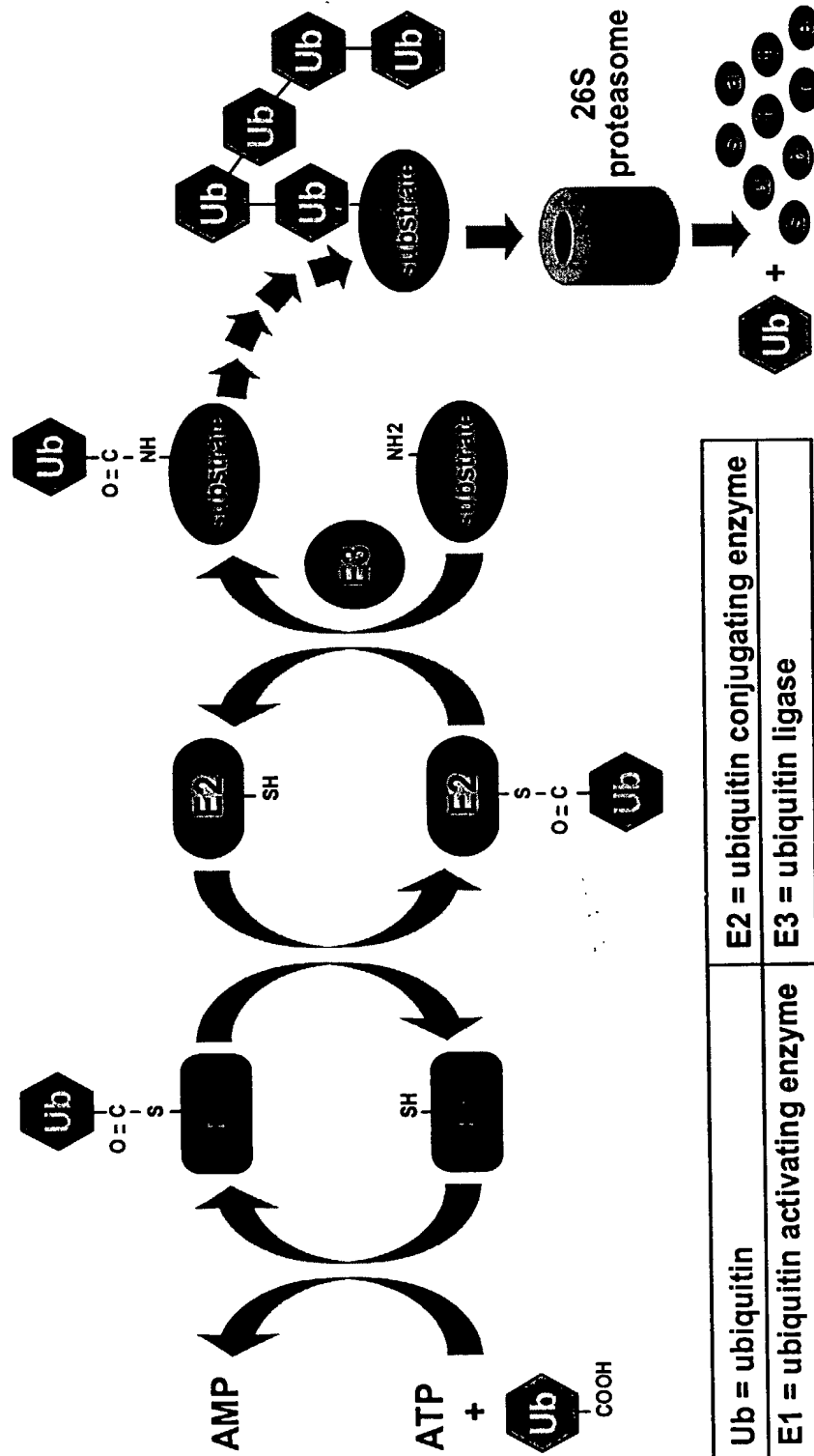
Figure 11A

A Reconstituted, Substrate-independent Assay for Studying Ligase Catalysis

Reaction Components

E1:

E2 (UbcH5): GST-fusion (cleaved), E.coli

E3 (Ring/cullin): His-tagged, coexpressed, baculovirus

Ubiquitin: FLAG-tagged, E.coli

Complete reaction

Reaction lacking:

E1 E2 E3 Ub ATP

FLAG-Ub$_N$

FLAG-Ub

ATP + FLAG-Ub

Roc1 Cul1 E2 — Catalytic core

Roc1 Cul1 E2 Ub Ub Ub Ub Ub Ub Ub

The substrate-independent reaction has the same catalytic properties and requirements for Roc1/Cul1 as the substrate-dependent reaction

Figure 11B

Bacterially-expressed TRAC1 for Ligase Activity Assay
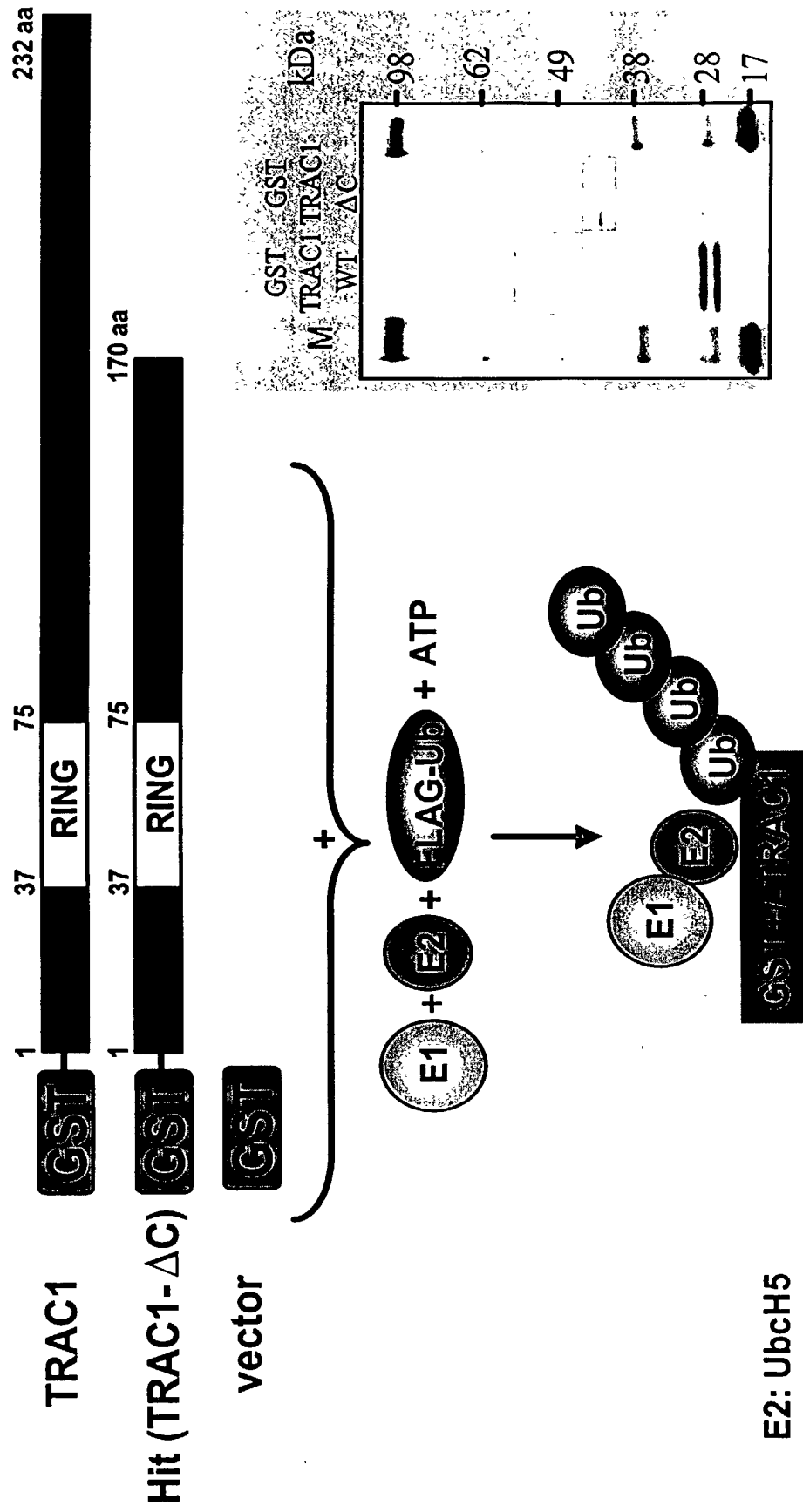
E2: UbcH5

Figure 12A

TRAC1 Exhibits E3 Ubiquitin Ligase Activity

Figure 12B
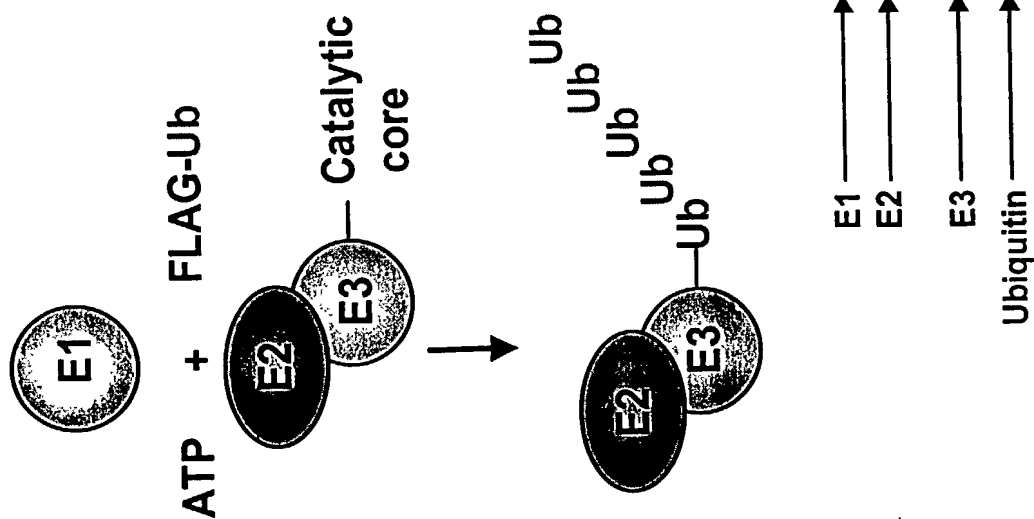
The Ring Domain is Required for TRAC1 Ligase Activity
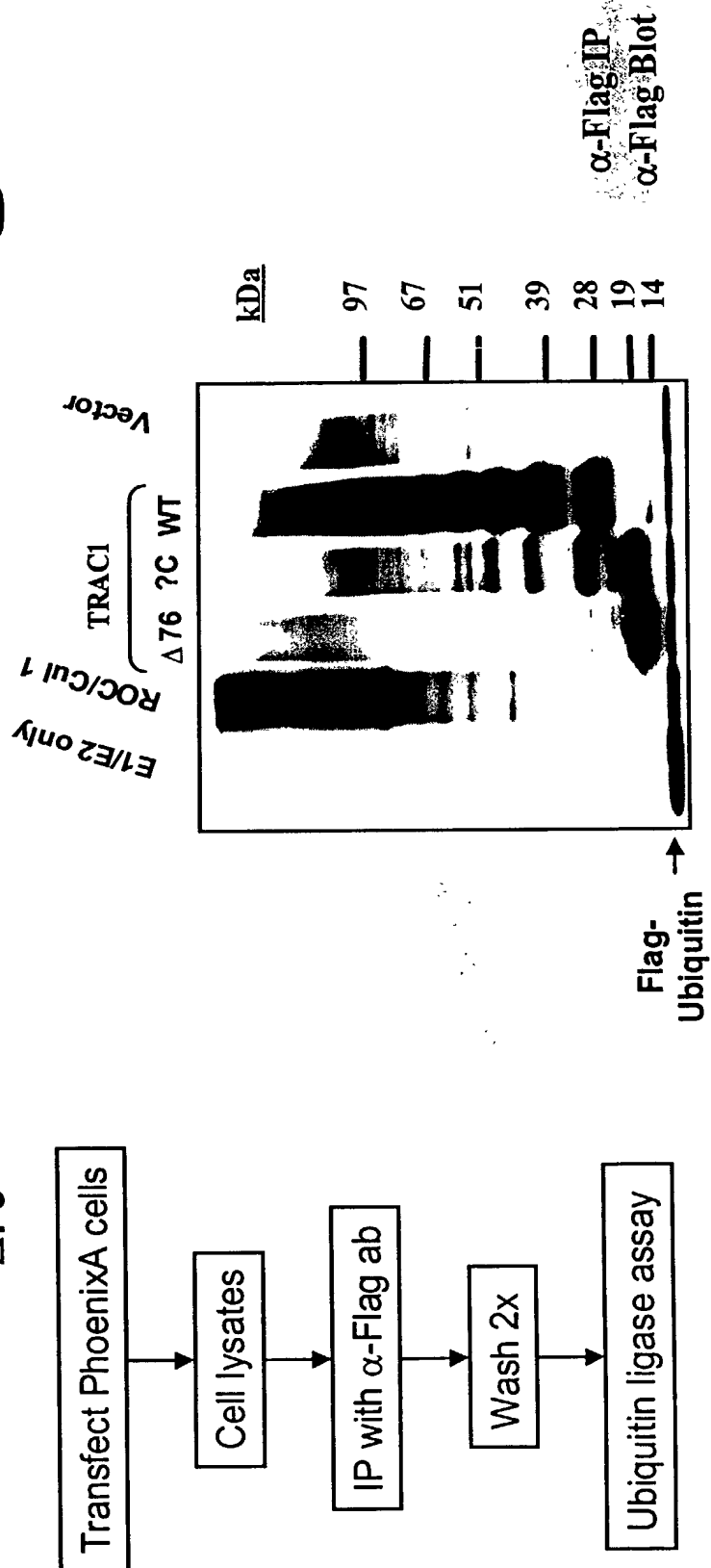
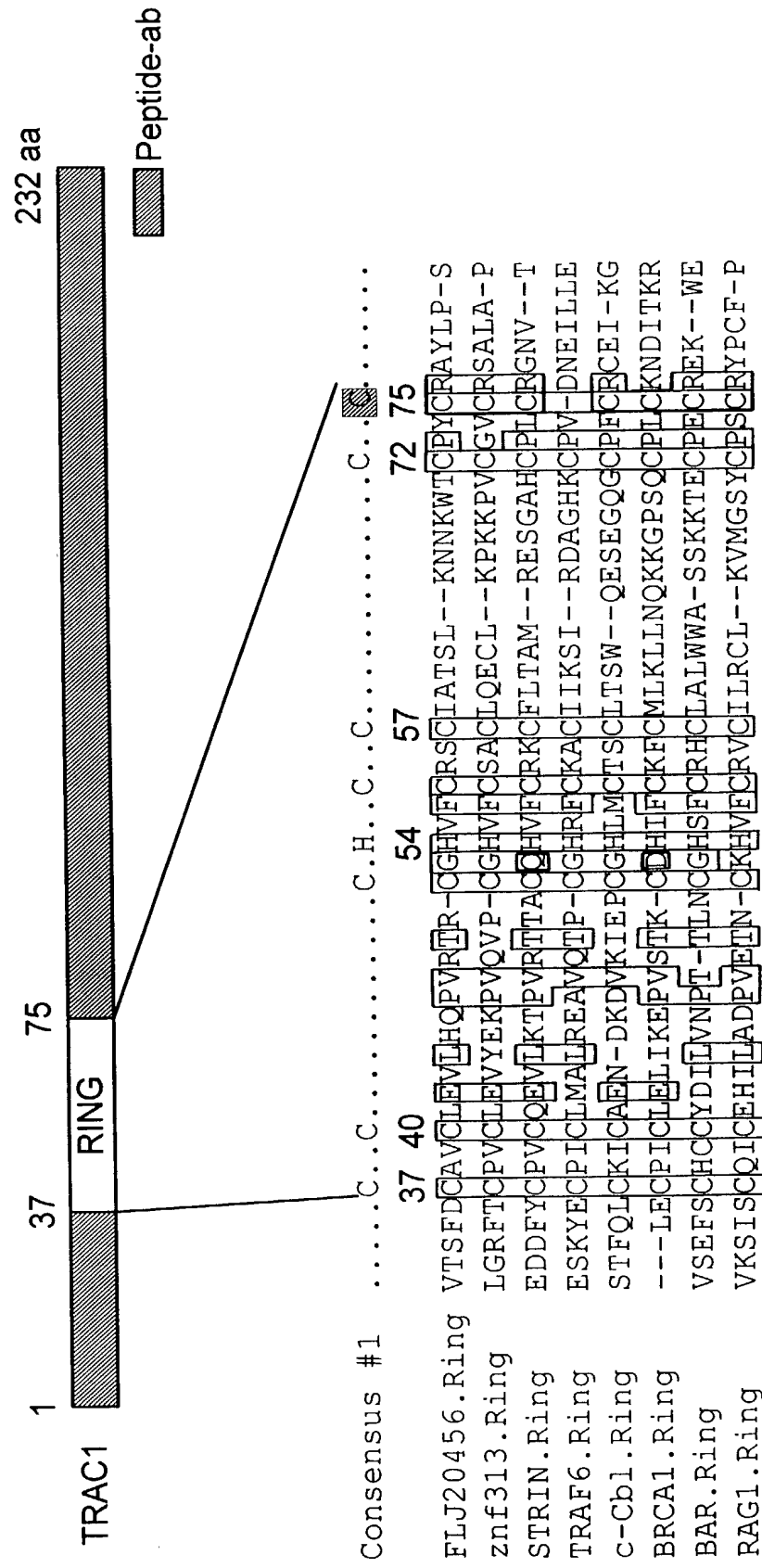# Point mutations in Conserved Cysteine Residues of the TRAC1 Ring finger Domain

TRAC1

1   37   75   232 aa

RING

Peptide-ab

```
Consensus #1   ......C.C..........C.H.C.......C.......
                   37 40           54  57        72 75
FLJ20456.Ring  VTSFDCAVCLEVLHQPVRTR-CGHVFCRSCIATSL--KNNKWTCPYCRAYLP-S
znf313.Ring    LGRFTCPVCLEVYEKPVQVP-CGHVFCSACLQECL--KPKKPVCGVCRSALA-P
STRIN.Ring     EDDFYCPVCQEVLKTPVRTTACHVFCRKCLTAM--RESGAHCPLCRGNV--T
TRAF6.Ring     ESKYECPICLMALREAVQTP-CGHRFCKACIIKSI--RDAGHKCPV-DNEILLE
c-Cbl.Ring     STFQLCKICAEN-DKDVKIEPCGHLMCTSCLTSW--QESEGQGPFCRCEI-KG
BRCA1.Ring     ---LECPICLELIKEPVSTK-CDHIFCKFCMLKLLNQKKGPSQCPLCKNDITKR
BAR.Ring       VSEFSCHCCYDIIVNPT-TLNCGHSFCRHCLALWWA-SSKKTECPECREK--WE
RAG1.Ring      VKSISCQICEHILADPVEIN-CKHVECRVCILRCL--KVMGSYCPSCRYPCF-P
```

- The following expression plasmids were generated:
  pEFnig/Ring finger point mutants: H54A, C75A, C37, 40A, H54C57A, C72, 75A
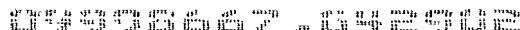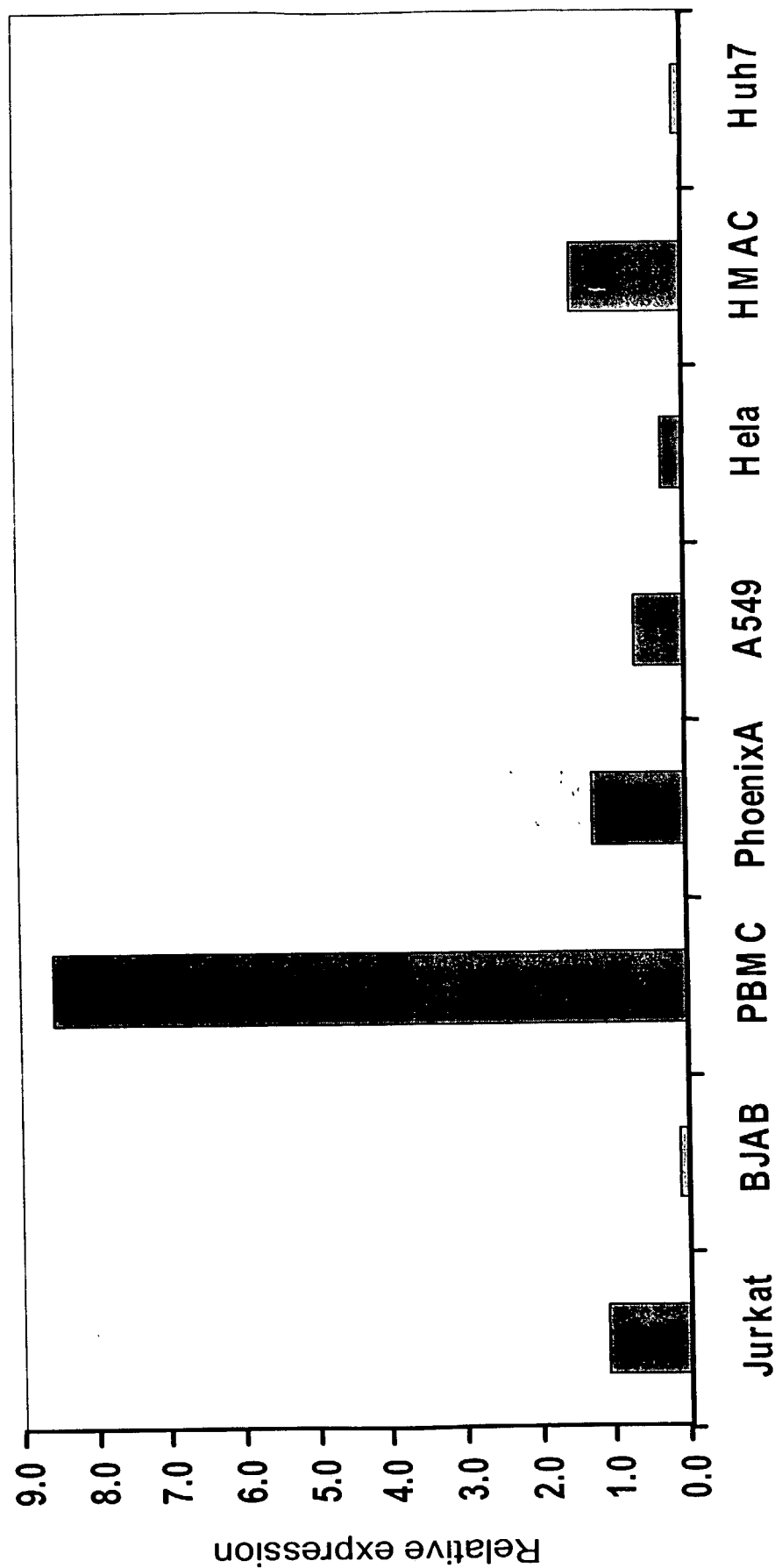 pEFnig/Myristylation site mutant: G2A

FIG. 13A.

Figure 13B

Point mutations in Conserved Residues of the TRAC1 Ring-finger Domain Disrupt Ligase Activity

Figure 14

Expression of TRAC-1 mRNA is ~8 fold higher in PBMC than in Jurkat cells

■ TRAC1/HuPO

Figure 15A

C-terminal Truncated TRAC1 Blocks
TCR-induced Ca2+ Influx
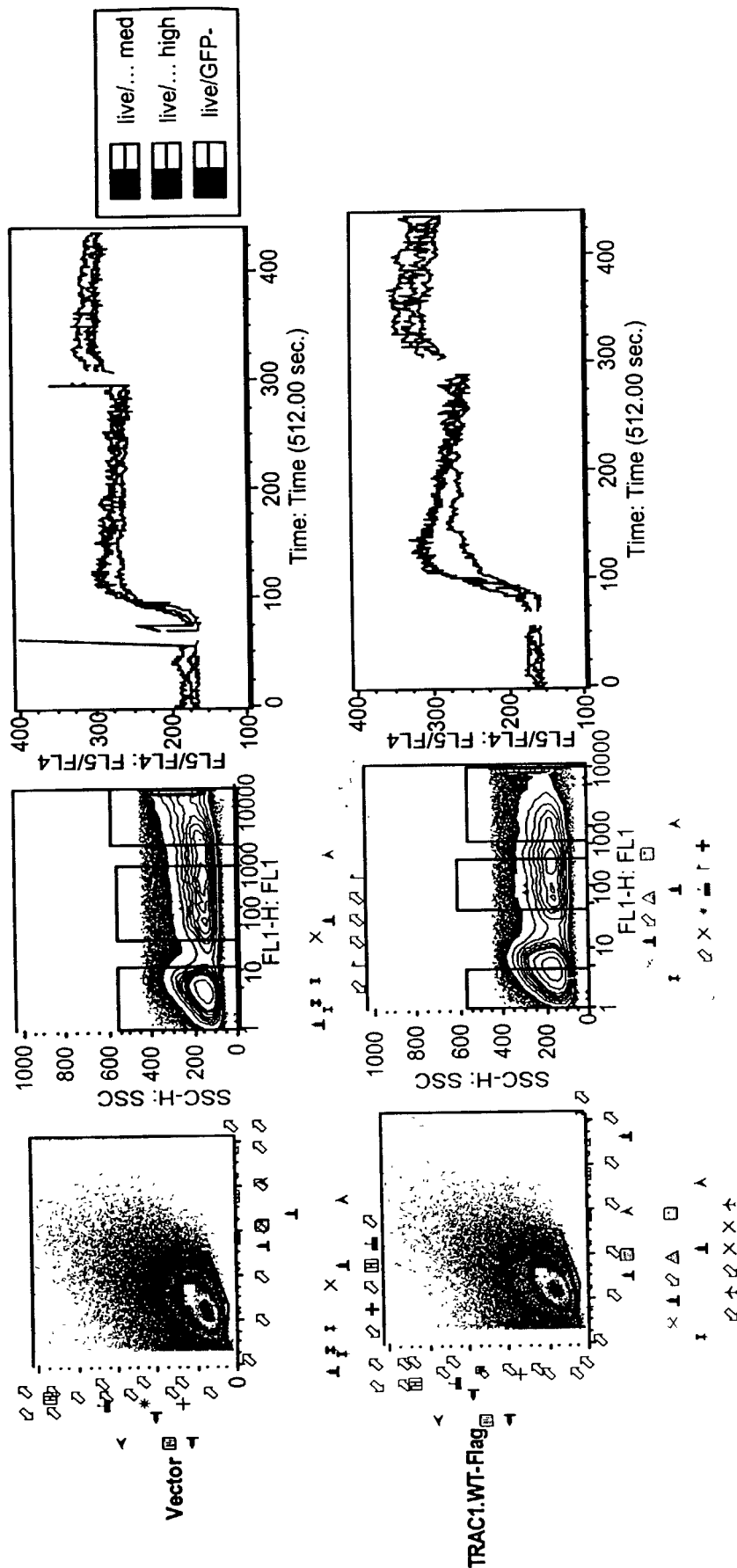
Figure 15B
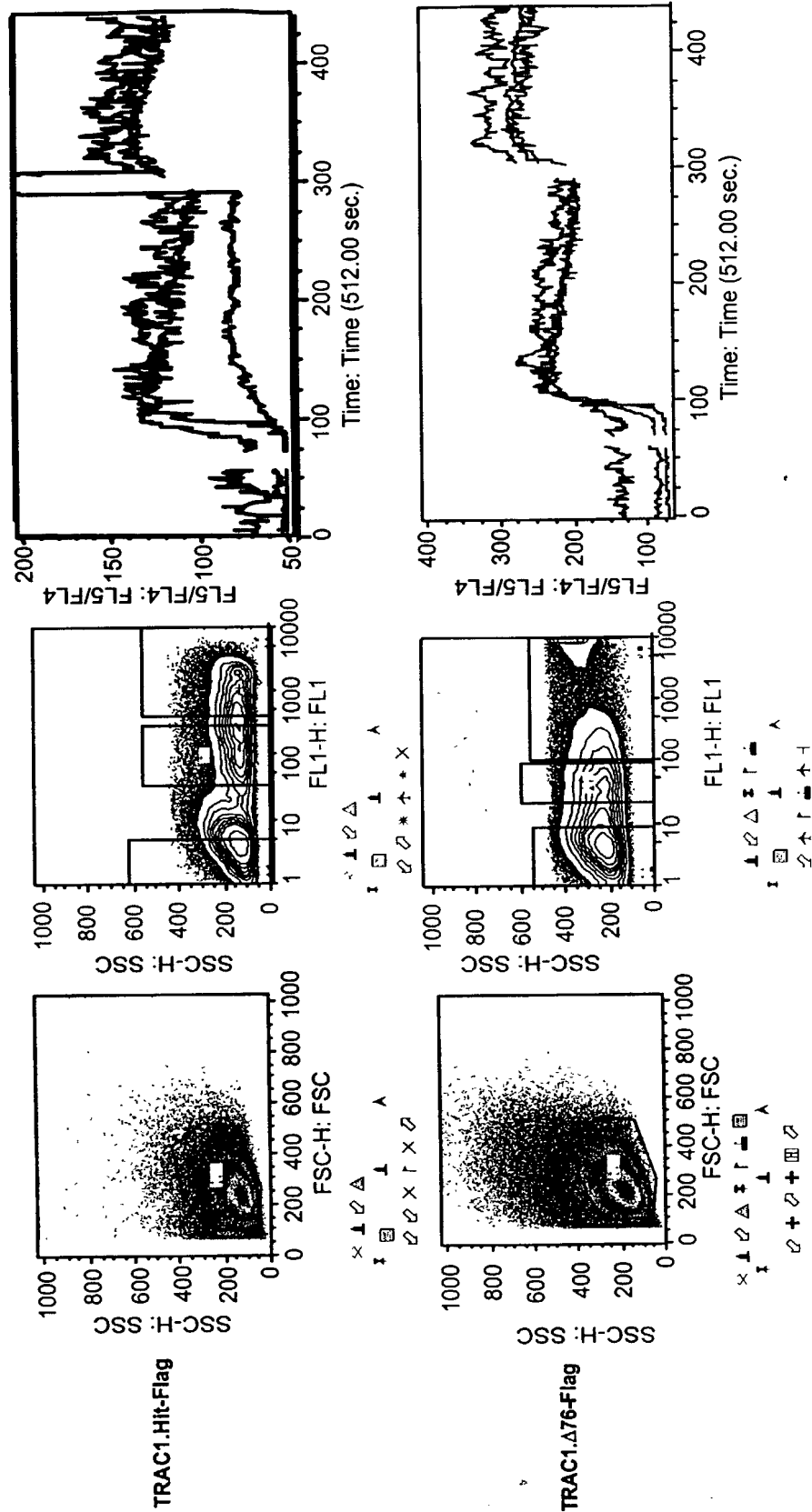
Figure 16

An Intact TRAC1 Ring domain is Required for Inhibition of α–TCR-Induced CD69 Up-regulation
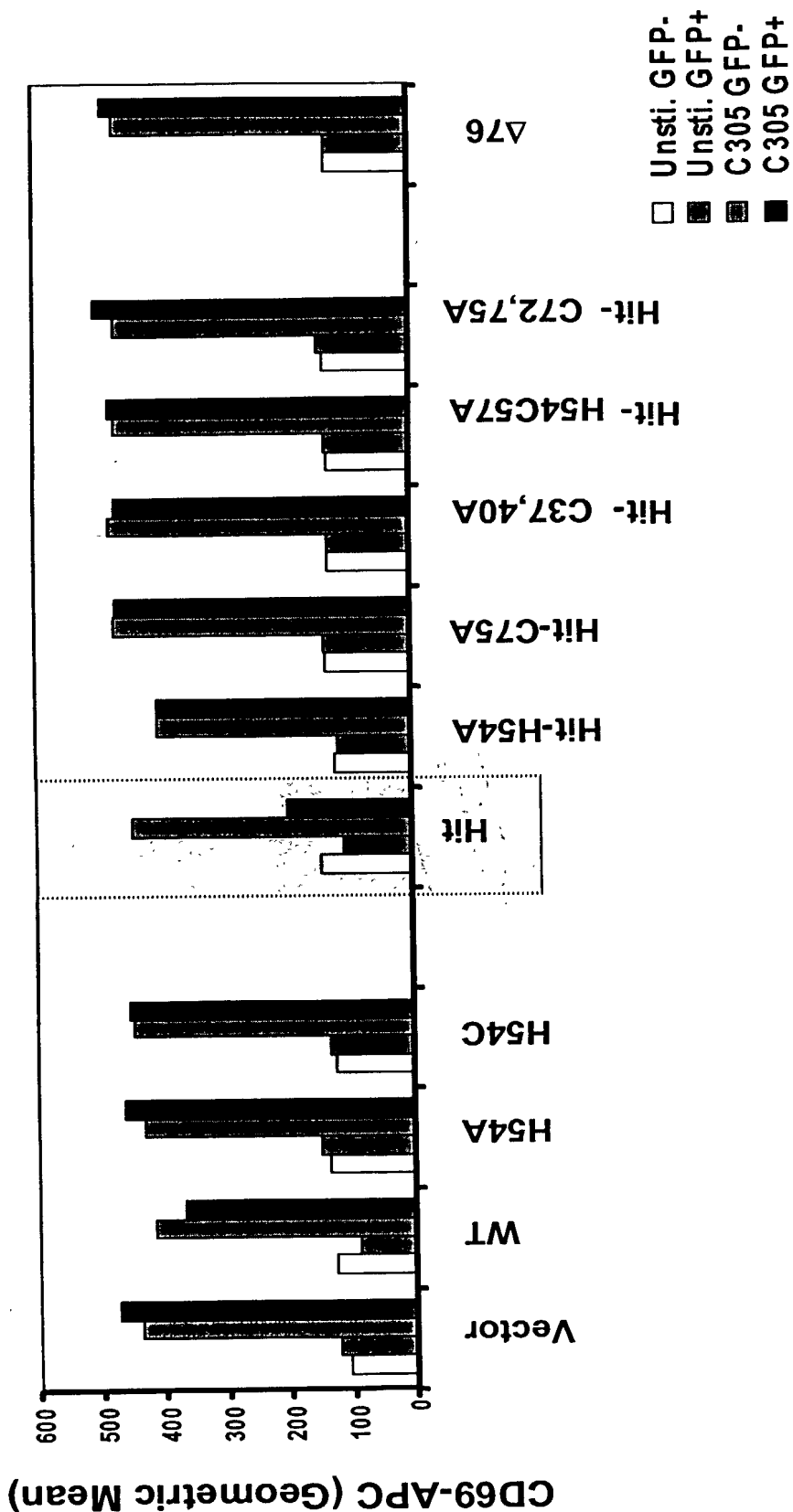

Legend:
☐ Unsti. GFP-
▨ Unsti. GFP+
▨ C305 GFP-
■ C305 GFP+

Y-axis (vertical): CD69-APC (Geometric Mean) — 0, 100, 200, 300, 400, 500, 600

X-axis categories: Vector, WT, H54A, H54C, Hit, Hit-H54A, Hit-C75A, Hit- C37,40A, Hit- H54C57A, Hit- C72,75A, Δ76

Figure 17

Summary of Functional Effects by Different TRAC-1 constructs

	Ubiquitin ligase activity	CD69 induction	Calcium mobilization
TRAC1	yes	→	+/− →
Hit	yes		
Δ76	no		
C37,40A			
C72,75A			
H54C57A			

Figure 18

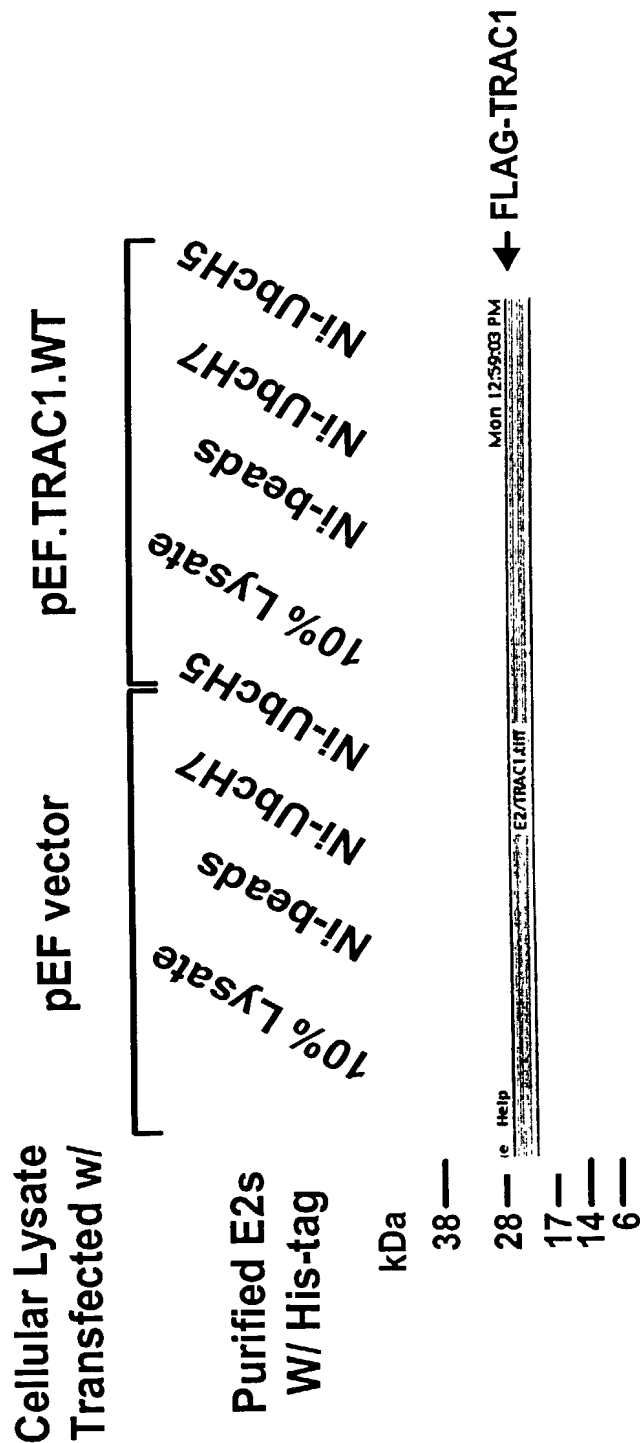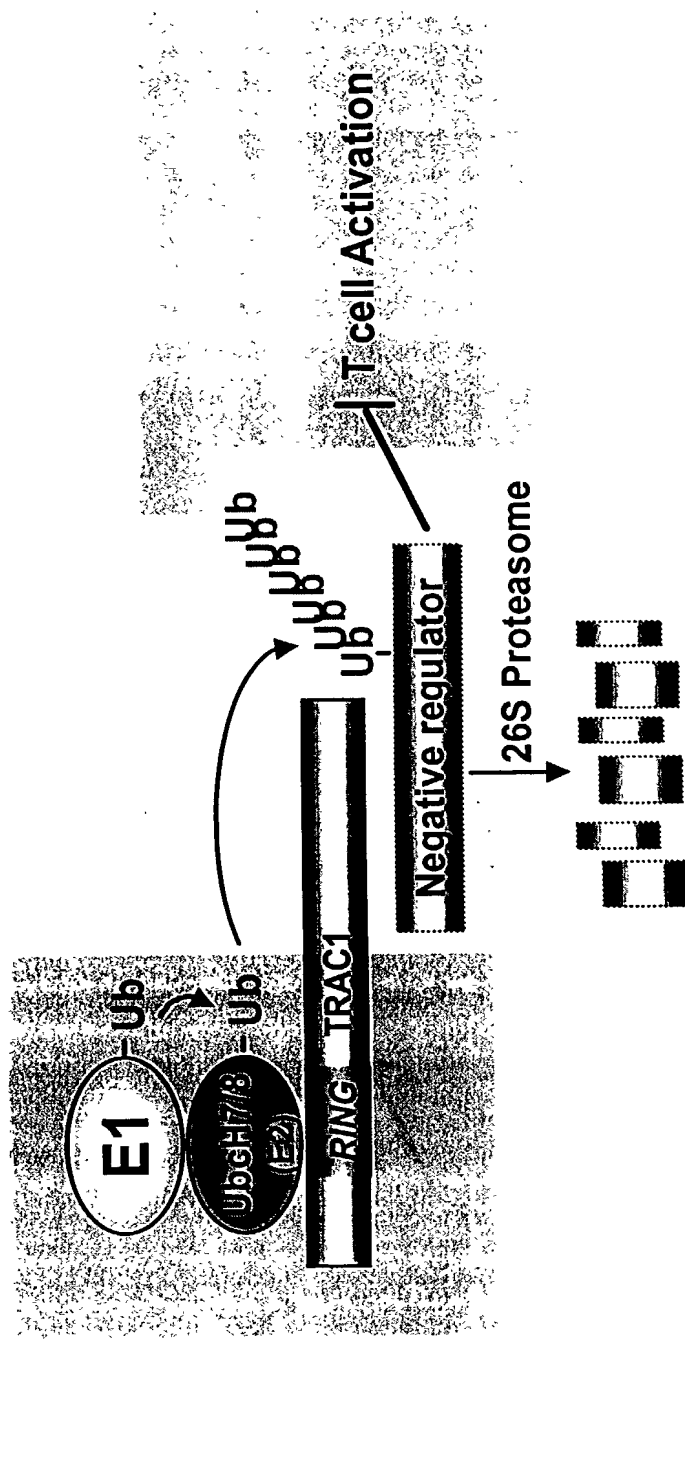# Transiently Transfected TRAC1 Protein Binds to Ubiquitin - Conjugating Enzymes (E2s) UbcH7 and UbcH5 *in vitro*

Figure 19

Model for TRAC-1 regulation of T cell activation